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MOLECULAR AND CLINICAL SEXUAL DIMORPHISM IN SYSTEMIC AUTOIMMUNITY

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Molecular and Clinical Sexual Dimorphism in Systemic Autoimmunity

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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“Books are not made to be believed, but to be subjected to inquiry. When we consider a book, we mustn't ask ourselves what it says but what it means.”

"Because learning does not consist only of knowing what we must or we can do, but also of knowing what we could do and perhaps should not do.”

-William of Baskerville
“The name of the rose” by Umberto Eco

ABSTRACT

Biological differences between the sexes of a species, also known as sexual dimorphism, can be found throughout developmental, physiological and pathological processes. In human disease, sexual dimorphism can explain marked differences in disease susceptibility. Rheumatic diseases, such as systemic lupus erythematosus (SLE) and primary Sjögren's syndrome (pSS), are chronic systemic autoimmune disorders that predominantly affect more women than men. Although many mechanisms have been put forward in order to explain this sex bias, the molecular underpinnings and their translation into disease phenotype are still not fully understood. Therefore, the aim of this thesis was to explore potential sex differences in genetic aspects that contribute to disease development, as well as to characterize clinical features that might exhibit a sexually dimorphic pattern.

In **Paper I**, we studied the expression of genes associated with pSS at basal state in splenic immune cells from wild type mice. The analysis revealed minor differences between female and male murine cells. Similar findings were obtained when human B cells and monocytes were investigated. Although these results suggested potential intrinsic differences, the extent of the sexual dimorphism observed in gene expression of risk loci could not entirely explain the marked sex skew in disease susceptibility.

To instead address differences in gene regulation rather than expression, in **Paper II** we examined whether single nucleotide polymorphisms (SNPs) associated with SLE and pSS could affect the gene expression in a sex-specific manner. The analysis resulted in identification of sex-specific expression quantitative loci (eQTLs) in human B cells. The study of sex-influenced eQTLs in other cell subtypes and in whole blood highlighted the context-dependent effect of these eQTLs.

Since variation in gene regulation of risk loci among the sexes can lead to a heterogeneous disease phenotype, in **Paper III, Paper IV** and **Paper V** we aimed to identify relevant sex differences in the clinical presentation of incident pSS, prevalent pSS and prevalent SLE, respectively. Our analyses showed that, despite being less prone to systemic autoimmune disorders, men have a more severe disease phenotype, characterized by more organ manifestations, an enhanced serological profile and more critical long-term complications when compared to their female counterparts.

In summary, our present work demonstrates the importance of sexual dimorphism in disease susceptibility and phenotype; also, it sheds light on possible molecular mechanisms that orchestrate the immune regulation of these complex disorders. Our results should raise awareness of relevant clinical sex differences that can aid in providing a tailored treatment to these patients.

RESUMEN

Las diferencias biológicas entre los sexos de una misma especie, también conocidas como dimorfismo sexual, influyen varios procesos biológicos. En humanos, el dimorfismo sexual es responsable de la marcada susceptibilidad para presentar alguna enfermedad o sintomatología. Las enfermedades reumatológicas son un exponente clásico del dimorfismo sexual. Las enfermedades reumatológicas, como el síndrome de Sjögren primario (SSp) y el lupus eritematoso sistémico (LES), son un grupo de padecimientos autoinmunes caracterizados por inflamación crónica y manifestaciones sistémicas, donde más del 90% de los pacientes son mujeres. Aunque ya se han propuesto algunos mecanismos fisiopatológicos, los procesos moleculares encargados del inicio de la enfermedad y las consecuencias de éstos a nivel clínico todavía no han sido completamente esclarecidos. Por tanto, esta tesis tuvo por objetivo estudiar aspectos genéticos que podrían contribuir al desarrollo de enfermedades reumatológicas de forma diferente entre los sexos; además de caracterizar e identificar manifestaciones clínicas que podrían afectar a los sexos con diferente frecuencia o severidad.

En el **Artículo I**, investigamos la expresión basal de genes que están asociados con el desarrollo del SSp y LES. El estudio se realizó primero en ratones, de los cuales se obtuvieron células inmunes del bazo. Los niveles de expresión génica entre las hembras y los machos no fueron considerables. En células inmunes humanas, la diferencia en expresión de estos mismos genes tampoco fue significativa entre mujeres y hombres.

Puesto que no encontramos diferencias notables en expresión génica, en el **Artículo II** examinamos si el dimorfismo sexual podría estar presente más en la regulación de la expresión de dichos genes. Para ello, analizamos si polimorfismos de nucleótido único (SNPs) serían capaces de influenciar la expresión de genes cercanos a estas mutaciones de forma diferente entre los sexos (eQTLs). El análisis reveló que, efectivamente, SNPs relacionados con SSp/LES actúan como eQTLs y son dependientes del sexo y del tipo de célula.

Las diferencias en el genotipo pueden tener diferentes efectos en el fenotipo de la enfermedad. En los **Artículos III-V,** encontramos diferencias clínicas significativas entre mujeres y hombres con SSp o LES. En general, los hombres con SSp y LES presentaron síntomas más severos que las mujeres.

El presente trabajo representa una aproximación a los mecanismos genéticos que difieren entre los sexos y podrían desencadenar un proceso autoinmune. Este estudio, además, muestra una imagen clínica distinta entre los sexos que podría servir para un mejor diagnóstico y manejo de las enfermedades reumatológicas.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. **Ramírez Sepúlveda JI**, Brauner S, Wahren-Herlenius M. *Sex-stratified expression of primary Sjögren's syndrome susceptibility genes in mice*. Submitted.

II. Lindén M, **Ramírez Sepúlveda JI**, James T, Thorlacius GE, Brauner S, Gómez-Cabrero D, Olsson T, Kockum I, Wahren-Herlenius M. *Sex influences eQTL effects of SLE and Sjögren's syndrome-associated genetic polymorphisms*. Biol Sex Differ. 2017;8:34.

III. **Ramírez Sepúlveda JI**, Kvarnstrom M, Brauner S, Baldini C, Wahren-Herlenius M. *Difference in clinical presentation between women and men in incident primary Sjogren's syndrome*. Biol Sex Differ. 2017;8:16.

IV. **Ramírez Sepúlveda JI**, Kvarnström M, Eriksson P, Mandl T, Norheim KB, Johnsen SJ, Hammenfors D, Jonsson MV, Skarstein K, Brun JG, the DISSECT consortium, Rönnblom L, Forsblad-d'Elia H, Magnusson Bucher S, Baecklund E, Theander E, Omdal R, Jonsson R, Nordmark G, Wahren-Herlenius M. *Long-term follow-up in primary Sjögren's syndrome reveals differences in clinical presentation between female and male patients*. Biol Sex Differ. 2017;8:25.

V. **Ramírez Sepúlveda JI**, Bolin K, Mofors J, Leonard D, Svenungsson E, the DISSECT consortium, Rantapää Dahlqvist S, Jönsen A, Bengtsson A, Rönnblom L, Sjöwall C, Gunnarsson I, Wahren-Herlenius M. *Sex differences in clinical presentation of systemic lupus erythematosus and lupus nephritis*. Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

AARDA	American Autoimmune Related Diseases Association
ACR	American College of Rheumatology
AID	Autoimmune Disease(s)
Alu	Alu element, from *Arthrobacter luteus*
AMA	Anti-Mitochondrial Antibodies
ANA	Anti-Nuclear Antibodies
AP2A2	Adaptor related Protein complex 2 Alpha 2 subunit
APC	Antigen Presenting Cell
APLN	Antiphospholipid Associated Nephropathy
BANK1	B-cell scaffold protein with Ankyrin repeats 1
BCR	B-cell Receptor
BILAG	British Isles Lupus Assessment Group
BLK	B Lymphocyte Kinase
C1q	Complement component 1, q subcomponent
C57BL/6	C57 Black Substrain 6 (mouse strain)
CCP	Cyclic Citrullinated Peptides
CD	Cluster of Differentiation
CD40L	CD40 Ligand
CXCL	C-X-C motif chemokine Ligand
CXCR	C-X-C motif chemokine Receptor
DISSECT	Dissecting disease mechanisms in three systemic inflammatory autoimmune diseases with an interferon signature (consortium)
DNA	Deoxyribonucleic Acid
dsDNA	double strand Deoxyribonucleic Acid
EGM	Extraglandular Manifestation(s)
ELISA	Enzyme-Linked Immunosorbent Assay
e.g.	“For example”, from the Latin *exempli gratia*
eQTL	expression Quantitative Trait Loci
ER	Endoplasmic Reticulum
ERα/ESR1	Estrogen Receptor alpha/Estrogen Receptor 1
ERβ/ESR2	Estrogen Receptor beta/Estrogen Receptor 2
ESSDAI	EULAR Sjögren’s Syndrome Patient Disease Activity Index
ESSPRI	EULAR Sjögren’s Syndrome Patient Reported Index
EULAR	European League Against Rheumatism
FAM167A	Family with sequence similarity 167 member A
FDFT1	Farnesyl-Diphosphate Farnesyltransferase 1
FDR	False Discovery Rate
FOXP3	Forkhead Box P3
GWAS	Genome Wide Association Study
H1N1	Hemagglutinin 1 Neuraminidase 1 (Influenza A virus)
HDL	High-Density Lipoprotein
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate Lymphoid Cell
IRF	Interferon Regulatory Factor
ISN/RPS	International Society of Nephrology/Renal Pathology Society
KIAA1542	See PHRF1
La	La Ribonucleoprotein domain family member 3
LN	Lupus Nephritis

LPS	Lypopolysaccharides
MG	Myastenia Gravis
MHC	Major Histocompatibility Complex
MS	Multiple Sclerosis
NET	Neutrophil Extracellular Trap
NK	Natural Killer
PAMP	Pathogen-Associated Molecular Pattern
PBL	Peripheral Blood Lymphocytes
PBMC	Peripheral Blood Mononuclear Cells
PHRF1	PHD and Ring Finger Domains 1
pSS	primary Sjögren's Syndrome
PXK	PX domain containing serine/threonine Kinase like
qPCR	quantitative Polymerase Chain Reaction
RA	Rheumatoid Arthritis
RF	Rheumatoid Factor
RNA	Ribonucleic Acid
Ro52	52 KDa Ribonucleoprotein
Ro60	60 KDa Ribonucleoprotein
SEM	Standard Error of the Mean
SLC39A8	Solute Carrier family 39 member 8
SLE	Systemic Lupus Erythematosus
SLEDAI	Systemic Lupus Erythematosus Disease Activity Index
SNP	Single Nucleotide Polymorphism
SS	Sjögren's Syndrome
SSA	Sjögren's Syndrome antigen A
SSB	Sjögren's Syndrome antigen B
STAT4	Signal Transducer and Activator of Transcription 4
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
Th	T helper cell
TIV	Trivalent Influenza Vaccine
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
TNIP1	TNFAIP3 Interacting Protein 1
TRIM21	Tripartite Motif containing 21
TROVE2	TROVE domain family member 2
WHO	World Health Organization

1 Background

1.1 Sexual dimorphism in human biology

Since early fetal development, women and men show characteristics that make them different and, at the same time, define them individually[1 2]. Differences between the sexes, defined as human female XX and human male XY, are found throughout a person's lifetime and are a quintessential part of their biological development, from an embryo until an elder.

Sexual dimorphism, defined as systematic differences between the different sexes of the same species, permeates and influences the function of many tissues, organs and systems in the human body. The human brain shows a distinctive sex-specific gene expression and sex-specific splicing pattern[3]; pain perception is different between women and men[4] and many studies have highlighted important sex differences in pharmacokinetics and pharmacodynamics[5], hinting that sex-specific therapeutic interventions could be of great benefit and preferable[6]. Sex differences are present also in our later years: aging occurs differently between the sexes[7]; women and men live longer and shorter lives, respectively. Even the most common causes of death between women and men are different[8].

Sexual dimorphism is also important to consider when we try to understand susceptibility to develop certain diseases. Cancer[9], cardiovascular disease[10-13], bowel disease[14], chronic lung diseases[15], osteoporosis[16], just to name a few, are diseases that are differentially represented between women and men. However, no other group of diseases are more heavily influenced by the sex of the individual than autoimmune diseases (AID)[17].

1.2 Sexual dimorphism in the human immune system

The sex of an individual can greatly influence the function of the immune system. The sexual dimorphism in the immunological response might serve different purposes and may respond to sex-specific external (environmental) and internal (physiological) factors. In other words, the immune system is tailored to offer protection in a sex-specific manner, depending on the particular situation of the individual (e.g. pregnancy vs menopause). This entails that our defense system is dynamic and is, thus, sensitive to changes that are acquired or inherent to human physiology. Several differences between the female and male immune system have been previously described. The disparities can be found either in infection susceptibility, innate or adaptive immune responses.

In terms of infection, men are more prone to develop bacterial[18 19], viral[20] and parasitic infections[21] than women aged between puberty and menopause. The enhanced female

resistance to infection constitutes the evidence for asseverating that the female immune response is more robust and, therefore, able to clear out infections at a faster rate than males, the later exemplified in the significantly lower risk for women to develop sepsis[22].

Sex differences in infectious diseases can be partly explained by differences in the innate immune compartment. Innate immunity represents the first line of defense against foreign antigens predominantly through pathogen-associated microbial pattern recognition (PAMP), such as the Toll-like receptors (TLR). TLRs are able to sense bacterial and viral components and provoke the stimulation of the cell in order to eliminate the infection; in the context of autoimmunity, TLRs are essential for understanding disease pathogenesis because a sustained or exacerbated TLR stimulation can lead to an overproduction of proinflammatory mediators[23 24]. Overall, many studies have highlighted the increased expression of genes from the TLR pathway and TLR7 in females as opposed to males[25]. For example, Berghöfer *et al*[26] demonstrated that peripheral blood lymphocytes (PBL) from women produce higher amounts of IFN-α after stimulation with a TLR7 ligand. Similarly, Griesbeck *et al*[27] showed that, upon TLR7 stimulation, human female pDCs produce higher amounts of IFN-α than their male counterpart. They suggest that this might be due to increased levels of IRF5 at basal state in females compared to males. The sex bias observed here is of great relevance, considering the association of augmented levels of type I interferon and female-preponderant systemic autoimmune diseases.

On the other hand, immune cells from males exhibit a different profile. PBMCs from men produce less IFN-α after TLR7 stimulation; however, upon TLR9 stimulation, they produce higher levels of the anti-inflammatory cytokine IL-10 than their female counterparts[28]. Moreover, male neutrophils have higher levels of TLR4 and produce more TNF than female neutrophils both at basal state and after stimulation with LPS, a TLR4 ligand[29]. The increased reactivity of male neutrophils to LPS and consequent increased secretion of proinflammatory cytokines could be a fair explanation for men's increased risk for septic shock. In mice, however, female neutrophils and macrophages have a higher phagocytic activity than in the males[30] and female APCs are better at presenting peptides than male APCs[31].

Cell frequencies can also be influenced by the sex of the individual. Abdullah *et al*[32] and Gleeson *et al*[33] reported higher numbers of NK cells in men, while Bouman *et* al[34] showed increased numbers of monocytes in men. Conversely, women are reported to have higher numbers of NK T cells[35-37]. In mice, type 2 innate lymphoid cells (ILC2) are reduced in female mice of an MS-prone model[38].

The human adaptive immune system also shows many signs of sexual dimorphism. Regarding sex differences in cell counts, scarce studies report higher numbers of T cells in women than men[34], and more specifically, higher counts in the CD4+ subset and CD4+/CD8+ ratio[39]. This sex skew in T cell numbers is mirrored by differences in function, as reported by Zhang *et al*[40], where CD4+ T cells from healthy women produced higher levels of IFN-γ as well as tended to proliferate more than CD4+ T cells from men. They also showed that stimulated male CD4+ T cells had a greater tendency for IL-17A production instead. In accordance with the previous study, a microarray of activated human CD4+ and CD8+ T cells identified significant differences in expression profiles between female and male cells[41].

The B cell compartment seems to be comparable between the sexes[42 43]. However, the concentration of serum immunoglobulins (Ig) differs between the sexes. IgA values are 20% higher in men than in women whereas IgM and IgG values are significantly higher in women than men[44]. The more robust humoral response observed in women may account for the higher levels of circulating Ig, which is prominently seen e.g. after administering a vaccine.

Response to vaccination differs between women and men[45 46]. As reviewed by Klein *et al*[47], women almost invariably respond with a higher humoral response against common vaccines such as influenza and hepatitis. A study has evaluated vaccination dosages and determined that women 18 to 49 years old vaccinated with a full dose of the trivalent inactivated influenza vaccine (TIV) responded as well or greater than men of the same age when vaccinated with a half dose[48]. Although the sex-biased mechanism driving the vaccination effect is still not clear, Klein *et al*[47] analyzed the difference in gene expression at different time points after yellow fever vaccination in a sex-specific fashion and identified that women significantly upregulate more TLR-associated genes that active the interferon pathway. Paradoxically, women presented a more severe 2009 H1N1 influenza infection than men[49 50] and were more reluctant to receive the A/H1N1 vaccine[51].

Constitutional and functional differences between a female and male immune system are generally overseen when analyzing experimental and clinical data. In terms of physiopathology, these differences could explain marked differences in disease susceptibility. While men mount a lesser immune response and tend to have more recurrent infections, women's greater immune responses put them at a higher risk for developing immunopathologies if homeostasis is not fully restored.

1.3 Sex bias in autoimmune disease susceptibility

According to the American Autoimmune Related Diseases Association, Inc. (AARDA), there are 80-100 different AID and an additional 40 are suspected of having an autoimmune basis. The increased susceptibility for females to develop AID is a well-documented and established notion in the field[17]. With the exception of ankylosing spondylitis[52], type I diabetes[53] and psoriasis[54] which exhibit a slightly higher frequency in men, the most commonly diagnosed AID show a marked female sex skew.

Hashimoto's thyroiditis, with an estimated female to male ratio of 10-20:1, leads the ranking of female-biased AID, followed closely by primary Sjögren's syndrome (pSS, 20-9:1) and systemic lupus erythematosus (SLE, 9:1). Most other common AID, such as multiple sclerosis (MS), rheumatoid arthritis (RA), dermatomyositis and myasthenia gravis (MG) exhibit a more modest ratio (2-3:1)[55].

Although women can be more commonly affected by AID, the onset and diagnosis of the disease can vary within this group. For example, pSS is usually diagnosed between 40 and 50 years of age whereas SLE is diagnosed most commonly between 25-35 years of age. This suggests that AID have different pathophysiological mechanisms that might be driven by several biological factors e.g. sex hormones, which fluctuate along a woman's lifespan. Actually, in childhood, where differences in sex hormone levels are not that striking, the female preponderance in rheumatic diseases is less than in adulthood[56]. This strongly suggests, then, a pivotal role of estrogen levels in disease onset.

Interestingly, the reproductive state of a woman can also influence the outcome of the disease. During pregnancy, it is widely accepted that AID dominated by T cell responses remit, whereas autoantibody-mediated AID tend to worsen. For example, in MS[57] and RA[58] the symptoms recede during pregnancy, although there can be an immediate flare post-partum. In contrast, SLE manifestations worsen during pregnancy[59 60] and there is an increased risk for transferal of disease-associated autoantibodies to the fetus, which may result in neonatal lupus with a potentially lethal cardiopathy[61].

1.4 Proposed mechanisms for female preponderance in AID

Despite the great body of research in AID, the female sex skew remains virtually elusive. There has been a major effort to identify factors that might predispose and lead to the development of AID; however, the complexity and variability of these chronic inflammatory diseases[62] pose a challenge when trying to find causalities.

Nevertheless, various mechanisms have been proposed in order to explain the higher frequency of AID in women. The major hypotheses are as follows, in no particular order of importance (**Figure 1**).

Genetics and epigenetics*:* Genome wide association studies (GWAS) have shed light on the genes implicated in several female-biased AID, including SLE[63-70], pSS[71], RA[72 73] and MS[74 75]. The studies show, not surprisingly, a dysregulation in immune-related genes in cases versus controls. Further elucidation of the cellular pathways in which these genes act and their genomic regulation could potentially explain the molecular mechanisms of these diseases. Also, sex differences in epigenetic regulation have been proposed[76]. These discrepancies might account for the enhanced hypomethylation of interferon genes observed in SLE patients in comparison with healthy controls[76-78].

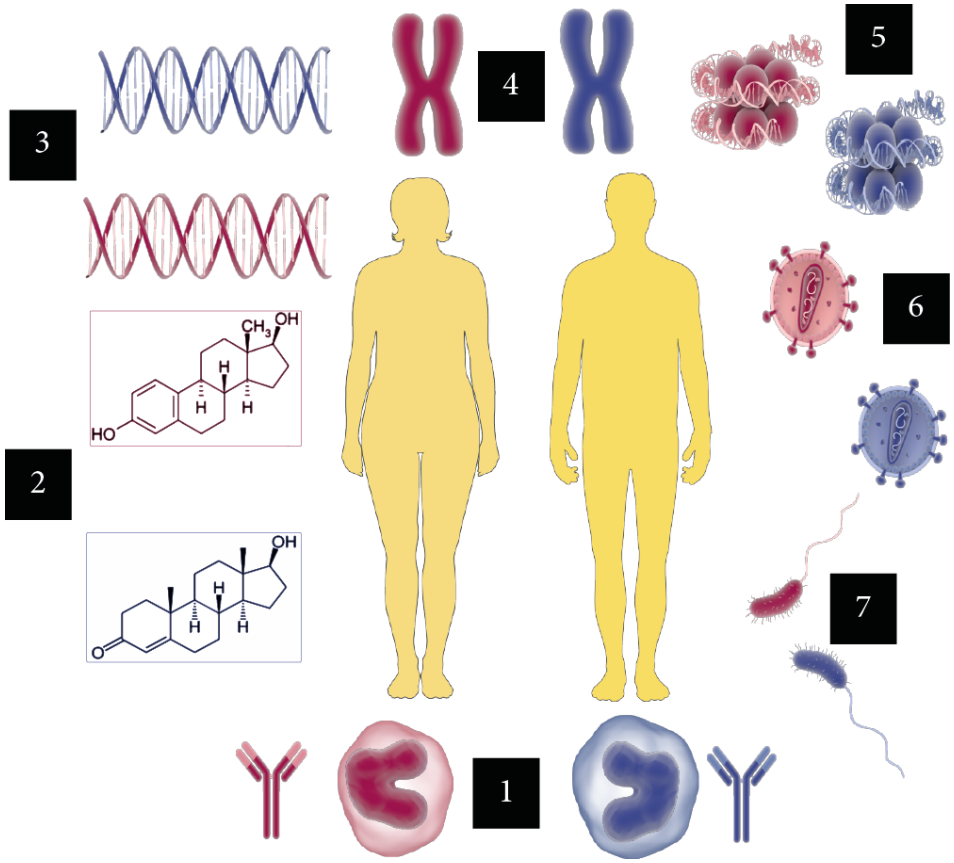


Figure 1. Factors that can influence the development of autoimmune diseases. (1) Sex differences in the immune system at steady state. (2) Sex hormones. (3) Genetics. (4) X chromosome. (5) Epigenetics. (6) Environmental factors (e.g. viral infections). (7) Microbiota.

X chromosome*:* For long, the X chromosome has been associated with the development of female-biased AID but, strikingly, is often disregarded in GWAS and other genetic studies. The human X chromosome contains many genes that are crucial for immune regulation, such as *TLR7*, *TLR8*, *CD40L* and *FOXP3*, among others. The link between AID

and the X chromosome could be explained through the double dosage effect, a consequence from an incomplete X-inactivation of the second X chromosome in women. It is believed that up to 15% of the genes in the second X chromosome escape inactivation, contributing thus to an overexpression of genes that are closely related to the immune system[79]. Not only are X chromosomes from AID patients more transcriptionally active[80] but also having X-chromosome aberrations can be associated with AID development. In women with SLE and pSS, the prevalence of trisomy X (47 XXX) is increased in comparison with healthy women. Similarly, Klinefelter's syndrome (47 XXY) is overrepresented in men with SLE and pSS[81] [82]. Additionally, the X chromosome contains around 10% of the described microRNAs in the human genome, many of which have been associated with regulation of the immune response[83-85]. This entails that microRNA-mediated mechanisms might vary between women and men due to differential X chromosome expression.

***Sex hormones**:* The apparent opposite effects of estrogen and testosterone are believed to be a major factor for understanding the sex bias in AID. While estrogens are considered predominantly immunostimulatory[86], testosterone has been related primarily to immunosuppression[87]. There's a large body of evidence that demonstrates the close interplay of sex hormones and the immune system[21 88 89]. Firstly, all major immune cell subsets express receptors for estrogen[90], represented by estrogen receptor alpha (ERα/ESR1) and estrogen receptor beta (ERβ/ESR2), which means that they are sensitive to fluctuations in estrogen levels. Estrogen seems to have a dual effect: at high concentrations (e.g. pregnancy) estrogens inhibit the Th1 pro-inflammatory pathways and stimulates the Th2 anti-inflammatory pathways; at low levels (e.g. menopause) estrogens stimulate the production of pro-inflammatory cytokines such as TNF-α and IL-1β. The complex immune regulation of estrogen can be achieved through the surface estrogen receptor or as a transcription factor via direct binding to estrogen response elements in the promotor region of many genes. As reviewed by Hughes[91], progesterone may have a biphasic behavior. At low physiologic levels, progesterone might be able to promote IFN-α, a main driver of AID. However, at higher concentrations, progesterone may suppress Th1 and Th17 responses by inducing the production of anti-inflammatory molecules. Additionally, elevated levels of prolactin have been associated with AID and correlated with serositis and anemia in SLE patients[92].

Regarding the primary male sex hormone, testosterone, the vast majority of the studies agree that androgens exert an immunosuppressive role, which in turn, is beneficial for prevention of AID. Interestingly, a retrospective study showed that decreased levels of androgens due to hypogonadism can increase the risk for developing rheumatic disease, namely RA and SLE[93].

***Environmental factors**:* Exposure to different environmental factors between women and men have been put forward as an added factor for developing AID. These factors can range from infectious agents[94], chemicals e.g. those found commonly in cosmetics[95], drug exposure[96 97]; pesticides have been associated with development of autoantibodies[98] and exposure to insecticides has been related to presence of anti-nuclear antibodies[99 100]. Barragan *et al*[101] have also found an association of exposure to organic solvents with autoimmune traits. Vitamin D has been implicated in the physiopathology of AID[102] since lower concentrations of this immunosuppressive vitamin could render an individual more susceptible to develop AID[103]. The exposure to different environmental factors might follow a gender role pattern due to differing occupations and behaviors between women and men. This type of studies are hard to perform due to their retrospective nature and the limitations in terms of measuring exposure.

***Microbiota**:* In recent years, the dysbiosis of the gut microbiota has garnered considerable attention due to its implication with the development of AID[104]. Many studies in mice have identified differences in bacterial species between females and males[105-108]. Although less progress has been done in humans, Davenport *et al*[109] have used a GWAS approach to study the human gut microbiome. They found that several bacterial taxa showed a sex-specific distribution and that some of them were consistent throughout the year's seasons. The link between sex-specific dysbiosis in humans and AID development is still to be clarified.

1.5 SLE and pSS: prototypical female-biased systemic AID

SLE is an AID characterized by multi-organ involvement, dysregulated autoantibody production[110] and elevated levels of type I interferon[111]. The incidence and prevalence of SLE vary considerably among countries and ethnicities[112]. In Sweden, for instance, the incidence is 4.8 cases per 100,000 inhabitants/year[113], while prevalence ranges from 79-144 per 100,000 in women and 12-25 per 100,000 in men[114].

SLE diagnosis is attained based on specific classification criteria. Currently, the most widely used diagnosis scheme is the 1982 American College of Rheumatology (ACR) classification criteria[115]. Briefly, the ACR criteria include 11 different domains: *malar rash, discoid rash, photosensitivity, oral ulcers, arthritis, serositis, renal disorder, neurologic disorder, hematologic disorder, immunologic disorder* and *antinuclear antibodies*. SLE diagnosis is achieved when a case fulfills at least 4 out of these criteria.

SLE is an insidious disease, with a heterogeneous course and spontaneous flare-ups. The Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) was developed to

assess disease activity and organ damage[116]. The SLEDAI scoring system considers 24 different descriptors, each with their own score weight. Thus, the SLEDAI total value correlates positively with disease severity. The British Isles Lupus Assessment Group (BILAG) disease index[117] is also commonly used and, in contrast with SLEDAI's global disease score, this scheme distinguishes and reports manifestations in separate organs/systems: *general, mucocutaneous, neurological, musculoskeletal, cardiorespiratory, vasculitis, renal* and *hematological*.

Among SLE organ manifestations, renal involvement stands out due to its association with increased morbidity and mortality in SLE patients[118 119]. Lupus nephritis (LN) can lead, after some years, to end-stage renal disease, a critical complication that affects between 10-30% of the patients[120]. LN has a diverse etiology, but is mainly associated with deposition of immune complexes containing anti-dsDNA[121 122] and/or anti-C1q antibodies[123], as well as genetic factors[124 125].

Histological examination of lupus nephritis is of utmost importance for effective therapeutic intervention. For this purpose, a histopathological classification has been devised. Renal biopsies are studied for lupus nephritis diagnosis based on either the World Health Organization (WHO) criteria[126] or the International Society of Nephrology/Renal Pathology Society(ISN/RPS)[127]. Lupus nephritis renal biopsies can be classified based on their mesangial, endothelial or epithelial patterns[127]. Also, a subset of SLE patients can also exhibit features of antiphospholipid syndrome, giving rise to a more severe form of renal involvement denominated antiphospholipid associated nephropathy (APLN)[128 129].

Another female-biased AID is pSS. pSS is a chronic systemic AID characterized by lymphocytic infiltration in exocrine glands, predominantly the salivary and lacrimal glands, resulting in a decrease in secretory function that leads to the hallmark symptoms of xerostomia (dry mouth) and xerophtalmia (dry eyes)[130].

pSS is considered the second most common systemic AID, after RA. It has been estimated that the prevalence of pSS ranges from 0.9 to 6 per 1,000 individuals[131]. Although not life threatening in its milder form, pSS can have a great negative impact on the quality of life of the patients and, in some instances, lead to severe systemic manifestations and lymphoma.

Besides the ocular and oral symptomatology, patients with pSS can present with many types of autoantibodies in serum, which serve both as biomarkers for diagnosis and as potential drivers of pathogenesis. Anti-Sjögren's-syndrome-related antigen A (SSA) and anti-Sjögren's-syndrome-related antigen B (SSB) are the most commonly associated with pSS. However, patients can also have anti-nuclear antibodies (ANA), anti-mitochondrial

antibodies (AMA), anti-centromere antibodies, anti-smooth muscle antibodies, antibodies against cyclic citrullinated peptides (anti-CCP), antibodies against carbonic anhydrase, anti-muscarinic receptor antibodies, rheumatoid factor (RF) and cryoglobulins[132 133].

Anti-SSA and anti-SSB, present in about 40%-80% of the patients, are the prototypical pSS autoantibodies and those most commonly used in clinical practice for patient classification. Anti-SSA, also known as anti-Ro (anti-Ro52 and anti-Ro60) are two antibodies that recognize a 52 kDa and a 60 kDa intracellular RNA-complex protein, respectively[134]. On the other hand, anti-SSB (anti-La) recognizes a 48 kDa ribonucleoprotein which is, in part, associated with the Ro particle (60 kDa Ro plus hY RNA)[135]. In consequence, anti-La antibodies are usually also found in sera containing anti-Ro60.

Although the predilection for generating antibodies against these intracellular antigens is currently not well understood, a major effort has been done to define the function of these autoantigens. The protein Ro52, alternatively named TRIM21, is an E3 ligase that negatively regulates proinflammatory cytokine production by ubiquitinating transcription factors of the interferon regulated factor family[136]. Noteworthy, antibodies against Ro52 are detected in patients many years prior to diagnosis, suggesting a gradual and chronic development of the disease and hinting at a possible pathogenic role for the autoantibodies in terms of tissue damage[137].

The Ro60 protein, encoded by *TROVE2*, is part of a ribonucleoprotein complex that, similarly to La, recognizes short noncoding Y RNAs. Additionally, Ro60 binds an RNA motif derived from endogenous Alu retroelements (retrotransposons). Alu transcripts are induced after stimulation with type I interferon and promote the secretion of proinflammatory cytokines by human peripheral blood cells[138].

The La antigen is a protein associated with newly synthetized RNA polymerase III transcripts. Its role is to stabilize these transcripts from exonuclease digestion[139]. Antibodies against Ro antigens can be found independently from anti-La antibodies, but not the opposite, and there is thus a high concordance of anti-La with anti-Ro positivity[140].

As previously exposed, autoantibodies play an important role in pSS. Actually, antibody positivity has been associated with disease activity in pSS[141]. However, the presence of autoantibodies represents just a fraction of the whole pathological picture. When it comes to patient classification, many other elements come into play. The most widely used consensus from Vitali *et al* [142] takes into account several classification criteria for pSS diagnosis: *subjective criteria* (Item I: Ocular symptoms and Item II: Oral symptoms) and *objective criteria* (Item III: Ocular signs, Item IV: Histopathology, Item V: Salivary gland involvement and Item VI: Autoantibodies).

pSS diagnosis is attained when the patient presents with any four out of the six items, as long as either item IV or VI is positive or when three out of the four objective criteria are present. There are also directives for secondary Sjögren's syndrome and exclusion criteria for SS.

As is customary in systemic AID, pSS patients can present symptoms in organs other than the exocrine glands. Extraglandular manifestations (EGM) can range widely[143 144] and are the basis for assessing the EULAR Sjögren's syndrome disease activity index (ESSDAI)[145]. The ESSDAI covers different domains of organ involvement: *constitutional* (recurrent fever, night sweats, weight loss), *lymphadenopathy/lymphoma* (enlarged lymph nodes, splenomegaly, B-cell malignancies), *glandular* (swelling of major salivary glands), *articular* (arthritis, synovitis), *cutaneous* (erythema, vasculitis, purpura, ulcers), *pulmonary* (persistent cough, interstitial lung disease, shortness of breath), *renal* (interstitial nephritis), *muscular* (myositis), *peripheral nervous system involvement* (polyneuropathy, mononeuritis), *central nervous system involvement* (multiple sclerosis-like syndrome, cerebrovascular accident, seizures, myelitis, meningitis) and *hematological* (anemia, neutropenia, thrombopenia, auto-immune cytopenia).

pSS belongs to the group of AID that is characterized by an exacerbation of the humoral response, leading to a chronic inflammatory state driven most likely by type I interferons[146] and, in some cases, systemic organ dysfunction. Although these clinical features are shared between female and male patients, it is still to be determined whether there are significant differences in disease presentation between women and men affected by pSS.

1.6 Sex differences in clinical presentation of AID

The sexual dimorphism observed in AID susceptibility extends likewise to the clinical presentation of the disease. Despite the overwhelming female preponderance, men suffering from AID have been repeatedly reported to have a more severe disease and a worse prognosis than their female counterparts.

In SLE, men present more renal disease[147-149], serositis[148 150] and pleurisies[148]. Further, Andrade *et al*[151] have identified male sex as a strong predictor for poorer long-term prognosis due to accelerated damage accrual while Manger *et al*[152] reported male sex as a risk factor for increased SLE mortality. Male sex is also deemed in MS as a factor for accelerated disease progression[153] and a significantly higher prevalence of comorbidities[154] such as diabetes, epilepsy, depression and anxiety. Although less clear, sex differences in RA severity and extraarticular manifestations have been also described, where women are

more frequently affected by keratoconjuctivitis sicca and men with rheumatoid nodules and lung disease[155 156].

Regarding pSS, several studies have addressed sex differences in clinical presentation[157-164]. As reviewed by Brandt *et al*[165], some authors have identified differences in EGM and serological markers, with a focus on female prevalence. However, there's no clear consensus on whether male sex is associated with a more severe disease. Interestingly, the first patient ever described with having symptoms suggestive of Sjögren's syndrome was a 42-year-old man in 1892.

2 Aims of the thesis

Numerous studies have underlined the higher propensity for females to suffer from autoimmune disorders. The most striking sex differences are observed in rheumatic diseases, such as Sjögren's syndrome, systemic lupus erythematosus, autoimmune thyroid disease and scleroderma.

Even though the sexual dimorphism in systemic autoimmune disorders is broadly acknowledged, there is still a gap in understanding the underlying molecular mechanisms that drive it, as well as a comprehensive characterization of sex-specific features that could help with decision making in a clinical setting. Thus, this thesis aimed to:

- Elucidate differences in the expression and regulation of genes associated with prominently sex-biased diseases such as primary Sjögren's syndrome and systemic lupus erythematosus (**Papers I** and **II**).

- Define differences in clinical and biomedical presentation of autoimmune disease in women and men, focusing on primary Sjögren's syndrome and systemic lupus erythematosus (**Papers III, IV** and **V**).

3 Results and Discussion

3.1 No striking sexual dimorphism in gene expression of pSS-associated risk loci at steady state

Genetics are an integral contributor to the development of rheumatic diseases. Many large-scale studies have identified single nucleotide polymorphisms (SNPs) in specific regions of the genome associated with immune regulation in systemic autoimmune diseases such as pSS and SLE. Although we have been able to pinpoint the genetic variations that may lead to disease, the molecular consequences of these variants are still being investigated.

In the context of sexual dimorphism, one first approach would be to examine whether these genes associated with disease are constitutively expressed differently between the sexes; these variants could cause a downregulation or exacerbation of the constitutive gene expression and its subsequent role in immune function regulation. In **Paper I**, we therefore studied if the gene expression of established pSS risk loci is different in females and males at basal state.

Our experiments consisted in assessing the gene expression by qPCR of *Blk, Cxcr5, Fam167a, Il12a, Irf5, Stat4* and *Tnip1,* both in the whole organ (spleen) and in sorted CD19+ B cells, CD3+ T cells and CD11b+ monocytes from C57BL/6 wild-type mice. The whole-organ analysis did not reveal any sex-related differences in expression of the risk genes studied (**Paper I**, **Figure 1**).

In order to account for cell heterogeneity in the whole spleen, we then performed the analysis in sorted immune cells. In the cell subset analysis, female splenic B cells showed a significantly increased expression of *Stat4* (**Paper I**, **Figure 2A**), while male splenic T cells had an increased expression of *Cxcr5* in comparison to their female counterparts (**Paper I**, **Figure 2B**). We did not identify a significant sex-dependent gene expression in splenic monocytes (**Paper I, Figure 2C**).

We conducted an additional experiment in order to replicate our previous results. As shown in **Figure 3**, male splenic B cells showed a significantly higher expression of *Cxcr5* but not T cells as previously seen (**Paper I**, **Figure 3A**). The augmented *Stat4* expression of female splenic T cells detected before was not replicated in this experiment (**Paper I, Figure 3A**). To confirm the higher expression of *Cxcr5* in male lymphocytes, another experiment was performed with purified $CD19^+$ B cells and $CD3^+$ T cells from spleens of 6 male and 6 female mice (10-18 weeks old). The gene expression analysis confirmed a higher expression of *Cxcr5* in male $CD19^+$ B cells, while no difference in expression was observed for T cells (**Paper I, Figure 3B**).

Taken together, our murine experiments revealed few instances where gene expression was significantly different between females and males. Of interest, *Cxcr5* was consistently more expressed in male murine cells than in female cells. Polymorphisms in the CXCR5 gene have been associated with the development of non-Hodgkin lymphoma, a severe complication observed in SLE and pSS and reported to be more common in male patients[166].

We hypothesized that the use of inbred, genetically homogeneous animals would allow us to identify more easily subtle differences in gene expression. Although we did find some, we also noted variation in gene expression across different experiments. This discrepancy could be explained by other factors unrelated to inherent sex differences in the genetic background, such as estrous cycle in the female mice or fighting in the male mice. Another reason could be technical variation that could mask existent minimal differences in expression. The apparent comparable basal state between female and male mice denotes that, regardless of sex, the homeostasis of the immune system is maintained and both groups are, in principle, capable of mounting a similar response.

To expand our analysis, we investigated the expression of the same genes from a publically available dataset of sorted human $CD19^+$ B cells and $CD14^+$ monocytes[167]. Peripheral B cells from humans (**Figure 2**) did not show a marked sex-biased gene expression.

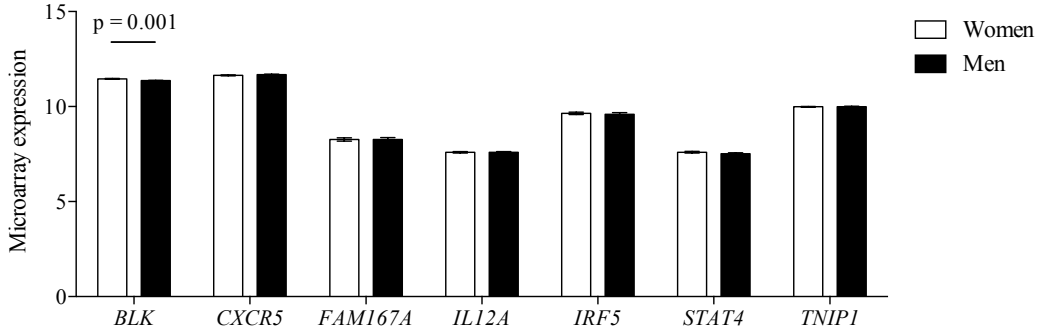


Figure 2: Expression levels of pSS susceptibility genes in human $CD19^+$ B cells. Expression levels of *BLK*, *CXCR5*, *FAM167A*, *IL12A*, *IRF5*, *STAT4* and *TNIP1* from sorted $CD19^+$ B cells. Results from 162 healthy women and 125 healthy men. Bars represent the mean expression of the risk loci in the specific group. The error bar indicates the standard error of the mean (SEM). Mann-Whitney U-test.

BLK was the only significant gene that was slightly more expressed in female B cells. Human monocytes (**Figure 3**) from women showed a small increased expression of *TNIP1* when compared with monocytes from men.

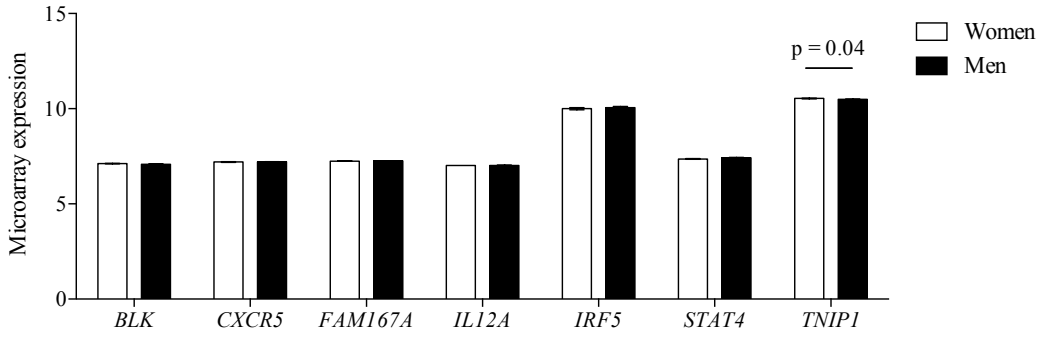


Figure 3: Expression levels of pSS susceptibility genes in human CD14$^+$ monocytes. Expression levels of *BLK*, *CXCR5*, *FAM167A*, *IL12A*, *IRF5*, *STAT4* and *TNIP1* from sorted CD14$^+$ monocytes. Results from 229 healthy women and 185 healthy men. Bars represent the mean expression of the risk loci in the specific group. The error bar indicates the standard error of the mean (SEM). Mann-Whitney U-test.

Contrary to the inbred mouse strains, the human population represents a broadly outbred group. Despite this stark contrast, the human immune cells still did not show a remarkable sexual dimorphic gene expression pattern, which in turn could be due to its genetic heterogeneity. These last results, however, reflect a fairer representation of the patient population and mirror the heterogeneity of systemic autoimmune disorders as well. Lastly, the comparable basal state gene expression between immune cells from women and men strengthens the notion that both sexes are equally equipped to respond; it's the cascade of events originating from that response that differs between them.

Although the analysis of steady-state gene expression was the scope of **Paper I**, it is worth noting that further studies will be needed to assess significant differences in gene expression upon stimulation. As many have reported before, female and male cells respond differently when immunologically challenged. The results shown here are from a healthy volunteer population, which means that the genetic background (and thus gene expression pattern) differs from disease-susceptible individuals. It would be informative to study how the variants associated with pSS have an impact on gene expression and if these variants affect the immune response differently between the sexes. This could be attained by studying samples from individuals carrying specific SNPs and then stimulating the cells to assess sexual dimorphic downstream effects.

3.2 SNPs associated with SLE and pSS influence the expression of neighboring genes in a sex-specific manner

SNPs can have several effects with regard to gene expression and regulation[168]. One of those outcomes is expression quantitative trait loci (eQTLs). These eQTLs are SNPs that influence the expression of genes in the vicinity (cis-eQTLs) or farther away (trans-eQTLs) from the polymorphic site[169]. Many SNPs previously identified in pSS[71] and SLE[170] GWAS are eQTLs. These eQTLs are context dependent e.g. cell-specific[167] and may be influenced by sex[171 172]. Considering sex as a variable in eQTL effects, in **Paper II** we therefore investigated whether SLE/pSS SNPs act as eQTLs in human $CD19^+$ B cells in a sample of 287 healthy volunteers (162 women and 125 men).

The SNPs investigated, shown in **Paper II Table 1**, were chosen from the current literature of genetic association studies in SLE and/or pSS, with the criteria of reaching genome-wide significance ($p<5.0*10^{-8}$) and being the first reported and/or top-associated SNP within the associated region, and/or being genotyped in the Fairfax *et al*. study[167], from whom we kindly obtained genotype and expression data.

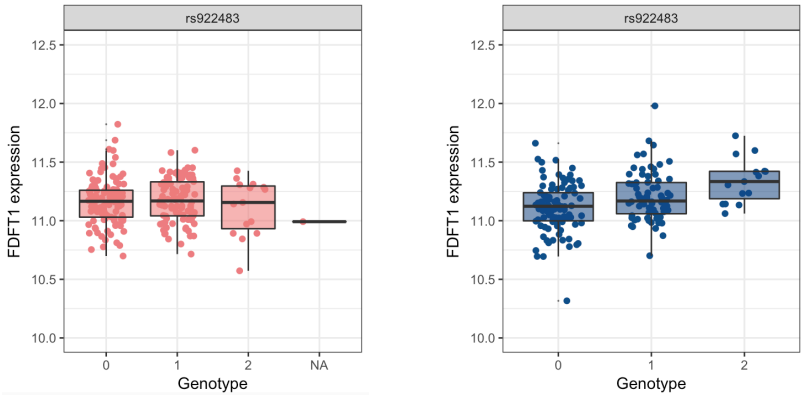
Our analysis revealed significant sex-specific eQTLs in B cells. The most relevant findings are summarized in **Paper II Table 3 and Paper II Figure 1,** and a representative scheme is provided in **Paper II Figure 2.** The most significant eQTL effect difference between females and males was found for rs4637409 (proxy SNP for rs10516487 in the *BANK1* locus) and the expression of *SLC39A8*. In females, presence of the SLE risk allele A was correlated with lower expression of *SLC39A8,* while the effect was opposite in males. The *SLC39A8* (Solute carrier family 39 member 8) gene encodes a manganese and zinc transmembrane transporter localized mainly at the cell membrane, but also at the lysosomal and mitochondrial membranes[173]. *SLC39A8* expression is under transcriptional control of the NFκB pathway. While its role in B cells has not been defined, it has been shown to be induced in other immune cells upon microbial challenge, leading to increased intracellular zinc levels[174]. In lung epithelium *SLC39A8* was found to be essential for zinc-mediated protection against stress-induced cytotoxicity at the onset of inflammation[175]. Reduced expression of *SLC39A8* in female carriers of the SLE-associated rs10516487 risk allele could potentially lead to enhanced inflammatory stress-induced cell damage and increased exposure of intracellular self-antigens.

The *CD74* gene encodes the class II invariant chain, and is well known for its function as a chaperone, which prevents binding of peptides to the MHC class II molecules in the endoplasmic reticulum (ER), promotes their exit from the ER, directing it into the

endocytic compartments, and contributes to peptide editing prior to antigen presentation[176]. CD74 has also been shown to be required for B cell maturation and function[177], and plays an additional role as an accessory signaling molecule on the surface of antigen-presenting cells[178]. In the present study, we found decreased *CD74* expression in B cells from females carrying the risk allele of rs10036748 (proxy SNP for the SLE and pSS associated rs7708392 in the *TNIP1* locus), which functionally may relate to an altered regulation of the peptide repertoire presented by MHC II. In males, the same allele was associated with increased *CD74* expression.

Regarding functional consequences of the *PXK* region SNP, one study has reported that it influenced the rate of BCR internalization, and that subjects carrying the risk haplotype had a decreased rate of BCR internalization, a process known to impact B cell survival and cell fate[179].

Paper II described sex-specific eQTL effects of disease-relevant SNPs in human B cells. Previously, the importance of conducting eQTL analyses in defined cell subsets has been pointed out since these eQTLs can be context dependent. To evaluate if the sex-specific eQTLs were exclusive to B cells, we interrogated the same list of SNPs previously selected (**Paper II, Table 1**) in a dataset of sorted human $CD14^+$ peripheral monocytes (414 healthy volunteers; 229 women and 185 men). **Figure 4** is representative of our main findings in monocytes.



SNP	Gene region	Gene name	p value	FDR	ß value
rs922483	*BLK/FAM167A*	*FDFT1*	0.0003	0.11	-0.12

Figure 4. Top (most significant) sex-specific eQTL identified in human $CD14^+$ monocytes. Genotypic effects of the proxy SNP rs922483 (r^2=1) for the pSS-associated rs2736345 (*BLK/FAM167A* locus) on expression of FDFT1. Genotype label represents the risk loci additive effect (0=homozygous for non-risk allele; 2=homozygous for risk allele; NA= Not available). Red box plots represent female monocytes while blue box plots indicate male monocytes.

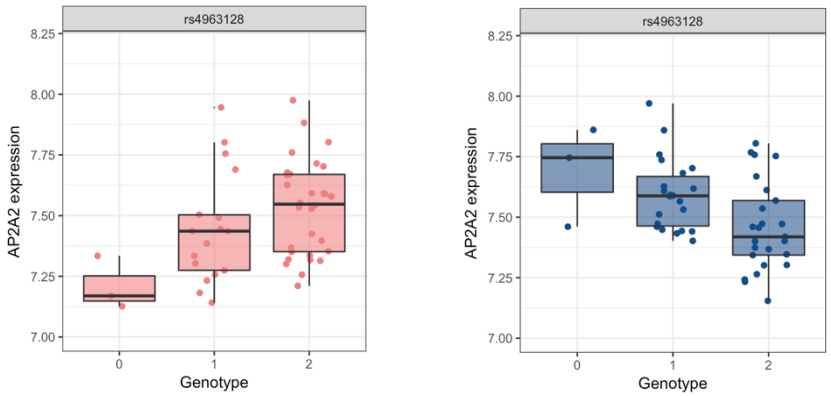
Interestingly, we indeed also found sex-specific eQTLs in monocytes; however, the top hits didn't match or were less significant than the ones found in B cells (**Paper II, Supplementary Table S3**). The most significant eQTL gene was *FDFT1*, which encodes

for an enzyme involved in cholesterol biosynthesis. *FDFT1* has been studied in the context of T2D and cardiovascular disease, where monocytes from patients with these diseases exhibit a dysregulation of lipid metabolism pathways[180]. In the C57BL/6.NOD-Aec1Aec2 mouse pSS model, defects in fat metabolism and fat deposition in salivary glands have been associated with pSS pathology[181 182]. In humans, serum lipid disturbances have been identified in pSS patients, with overall significantly lower levels of total cholesterol and HDL when compared to non-pSS sicca controls[183]. Additionally, fatty cell infiltration in salivary glands from pSS patients has been described[184], although its pathogenic role is still to be elucidated. According to our results, female individuals who have the risk allele have a modest reduction in expression of *FDFT1* but significantly reduced when compared to male individuals. Further investigation shall clarify the role of lipid metabolism in exocrine gland dysfunction and pathogenesis, as recent studies have underlined the connection of a faulty immune metabolism with the development of AID[185].

Although pSS and SLE are driven primarily by processes pertaining to the adaptive immunity compartment, there's increasing evidence suggesting a role for cells of the innate immunity in systemic inflammation. More specifically, neutrophils have garnered recent attention due to the role of NETs in the pathogenesis of SLE and various others autoimmune diseases[186]. Despite being uncommon, secondary autoimmune neutropenia can also be associated with other autoimmune diagnoses[187]. Importantly, neutrophils have been described as inducers of type I interferon production[188] but, notably, as synthesizers and secretors of type I interferons[189] and other proinflammatory products[190 191]. Taking into account that neutrophils are the most abundant cell type in circulation, that a recent study showed marked sex differences in gene expression[192], their counts are higher in women than men[193] and their pathogenic role in systemic autoimmunity, we further analyzed a dataset of sorted human neutrophils[194] (100 healthy volunteers; 50 women and 50 men) in order to assess sex-specific eQTLs.

As we expected, we were able to find sex-specific effects that were exclusive to neutrophils, and not present in the previous B cells and monocytes studies (**Figure 5**). The most significant sex-specific eQTL gene in neutrophils was *AP2A2*. *AP2A2* encodes for an adaptor protein involved in clathrin-mediated endocytosis[195]. Neutrophils can be activated by circulating immune complexes and environmental stimuli[196 197]. This activation is prominently mediated by TLRs, both in the surface of the cell and in the endosomal compartment. Indeed, human neutrophils express TLR1 through TLR10, except TLR3[198]. Interestingly, a study has shown that neutrophils are able to express TLR9 both in the plasma membrane and in the endosomal network[199]. In the context of systemic

autoimmunity, TLR9 is of relevance since its sustained engagement is directly associated with production of IFN-α[200 201] and an interferon signature is a hallmark for systemic autoimmunity.



SNP	Gene region	Gene name	p value	FDR	ß value
rs4963128	*IRF7/PHRF1/KIAA1542*	*AP2A2*	0.001	0.06	0.25

Figure 5. Top (most significant) sex-specific eQTL identified in human CD16^{+} neutrophils. Genotypic effects of the SLE-associated rs4963128 (*IRF7/PHRF1/KIAA1542* locus) on expression of *AP2A2*. Genotype label represents the risk loci additive effect (0=homozygous for non-risk allele; 2=homozygous for risk allele). Red box plots represent female neutrophils while blue box plots indicate male neutrophils.

In order for endosomal TLRs to encounter its ligands, the uptaken stimuli must be transported by vesicular trafficking into the corresponding endosomal compartment. As shown above, the increased expression of *A2P2* in female neutrophils which carry the risk SNP might hint at an enhanced vesicular trafficking, making TLR ligands, such as those for TLR9, more readily available and thus promoting a more robust neutrophil response, with the resulting proinflammatory effects. It is important to recognize that the sex-specific eQTL analysis performed in neutrophils represents a small study. However, its results might have interesting implications in understanding the intricate cell interaction observed in complex systemic diseases.

In summary, our results highlighted that SLE and pSS related SNPs have a wide effect as eQTLs in a cell-specific manner. Considering that these diseases are heavily sex biased and that these SNPs might coincide with already inherent sex-influenced eQTLs in the genome, we wondered if these sex-specific eQTLs could also be identified in less sex-biased autoimmune disorders. Therefore, our initial B cell analysis also contemplated an evaluation of T1D SNPs in the same dataset, as the sex difference in this autoimmune disease is very small. The results (**Paper II, Supplementary Table S4**) revealed that the T1D SNPs examined only exhibited a modest sex-specific effect; none of them were as significant or had a comparable size effect as the pSS/SLE SNPs.

The variation in sex eQTLs across cell types and diseases hints at an equally complex molecular mechanism. The possible mechanism of action of local eQTLs has been reviewed by others[169]. When sex is considered as a variable, the effect of the eQTL can have various outcomes[172]. Strikingly, a given SNP can have an eQTL effect in opposite directions between women and men. This last scenario suggests a more complex molecular mechanism that is not yet fully understood. However, we would like to propose two modes of action for sex-specific eQTLs.

When we consider the pSS/SLE SNPs that showed a significant sex-specific eQTL, most of them are located in non-coding or intergenic regions with regulatory activity. These polymorphisms could be responsible for repression or enhancement of gene expression by e.g. epigenetic regulation. Sex hormones, namely estrogen and testosterone, have DNA-binding capabilities and, thus, can act both as transcription factors and chromatin modifiers[202]. The recruitment of the regulatory machinery, due to epigenetic changes driven by the SNP, could have an amplifying effect on the genetic region neighboring the SNP. We hypothesize that this process might influence the expression of the genes in close proximity to the SNP on a sex-specific manner, based on the presence of sex hormones.

Another mechanism to understand sex-specific eQTLs comprises sex differences in gene regulation downstream of the risk loci studied. The SNP could directly affect the regulation of the risk gene which, in turn, could lead to differences in the expression of nearby genes through a pathway that is differentially regulated between the sexes, most probably orchestrated by sex hormones.

The mechanisms described above might represent an oversimplification of the intricate gene regulation that might be responsible for sex-specific eQTLs. It's important to note that, although these two processes could happen simultaneously, there might be many more possibilities, SNPs, eQTLs and stimuli that could explain the development of complex diseases such as SLE and pSS.

Lastly, the SNPs that have been identified in SLE and pSS GWAS represent susceptibility loci that are, most likely, particular to women, since they represent 90% of the studied population. This means that, although women and men might share genetic variants, there might also be variants that are specific to male patients that, due to low representation or exclusion in GWAS, are not being appropriately captured. While conducting sex-specific GWAS in heavily sex-biased diseases is essentially not feasible, some[203] have proposed methodologies to address this. Sex-specific genetic susceptibility

might shed light on molecular differences that might explain the heterogeneity in disease presentation between women and men.

3.3 Women and men with pSS present different clinical features at time of diagnosis

If we observe differences in the genotypic regulation between the sexes, then it could be expected to also find variation in the phenotypical presentation of a disease between the sexes. In fact, many studies have underlined that, despite women being more susceptible to develop systemic AID, men often present the same disease in a more severe form. In the case of pSS, some studies have shown varied results when it comes to defining sex differences in clinical presentation of the disease. The lack of consensus prompted us to study our own well-characterized cohort of pSS patients collected in the Stockholm area during 5 years[204]. In **Paper III**, we assessed the clinical and immunological profile of incident cases of female and male pSS patients in a population-based cohort (199 patients; 186 women and 13 men) to determine if the clinical and serological presentation of the disease differs between sexes, and replicated the findings in an independent cohort (377 patients; 368 women and 9 men).

In our exploratory Stockholm cohort, we did not identify significant differences in the presentation of glandular manifestations related to sicca symptoms (**Paper III, Table 1**). The major differences where observed in EGM, where men more often presented EGM; also, frequencies of pulmonary (interstitial lung disease) and cutaneous (vasculitis) manifestations were significantly higher in the male pSS group (**Paper III**, **Table 2**).

After measuring autoantibody levels in patient sera by ELISA, our most significant serological finding was that SSA+ male patients had higher levels of anti-Ro52 than SSA+ female patients (**Paper III**, **Figure 1**). Notably, Ro52 antibodies have been associated with pulmonary disease, particularly interstitial lung disease and pulmonary fibrosis [205 206], which we also observed more frequently in men from our cohort.

Although this finding can help explain the increased severity of the disease in men, the few sera available for analysis should be noted. Further studies of incident cases with larger sample sizes is needed to consolidate the serological difference between female and male patients. Serological investigations performed in previous studies of prevalent cases show considerable discrepancies between the frequencies of anti-Ro/SSA positivity among the male patients. The different observations may relate to the time point of serum sampling, as it is possible that more women develop higher autoantibody levels with disease progression.

The differences between studies could potentially also depend on the assay used for autoantibody testing and the presence of Ro60 and Ro52 antigens, respectively.

In order to validate our findings, we analyzed a similar cohort of incident pSS cases from the Rheumatology Clinic in Pisa, Italy. **Paper III**, **Table 4** recollects the features and EGM that significantly differed between female and male patients from the replication cohort, as well as the meta-analysis conducted to assess replicability of our results. Unfortunately, sera from the time of diagnosis were not available in this cohort.

A strength of our investigation is that the studied exploration cohort was generated in a population-based manner representing approximately 95% of all incident cases in the specific geographical area during the five year-period during which the cohort was established[204]. By this approach, selection bias was avoided. Also, more than 90% of the patients were examined and diagnosed by the same clinician, diminishing variation in assessment procedures and evaluation[204]. An additional strength of the study is that an independent cohort of incident pSS was used to verify observations. Around 90% of patients were seen by one specific clinician also in this cohort. Noteworthy, the replication cohort was not population-based, potentially explaining some of the differences observed between the cohorts. The catchment and inclusion of patients in the replication cohort might have been more delayed than in the exploratory cohort, which could account for higher numbers of EGM. Despite this, men from the replication cohort still showed a more severe disease phenotype.

More than nine out of ten patients with pSS are women, and an inherent weakness of studies of sex-differences of pSS is thus the smaller number of men in any given cohort. This is also the main drawback of the present study, despite including all cases in a densely populated defined geographical area during a five-year period and using a replication cohort.

3.4 Sexual dimorphism in pSS after long-term follow-up

Systemic inflammatory disorders often present a heterogeneous disease course throughout time. This means that the disease phenotype at diagnosis might significantly fluctuate due to spontaneous flares, comorbidities, treatment, disease duration, and so on. Since we observed important sex differences in the clinical presentation of pSS at time of diagnosis, in **Paper IV** we investigated if the frequencies of symptoms and manifestations notably differed between female and male pSS patients after many years of having their disease. Similar to Paper III, **Paper IV** assessed the occurrences of autoantibodies, clinical manifestations related to ESSDAI and other comorbidities common in pSS.

The data analyzed consisted of a large pSS cohort collected in several Scandinavian Rheumatology centers (DISSECT consortium). Our studied population comprised 899 female patients and 68 male patients (**Paper IV, Table 1**). We identified significant sex differences in terms of serological parameters and frequencies of some organ involvement. Overall, men presented distinctively with more severe clinical complications and positivity for a broader autoantibody repertoire.

Our serological investigation revealed that the humoral response between the sexes was different; particularly, men presented more often with La/SSB, Ro/SSA+La/SSB and ANA positivity (**Paper IV**, **Table 2**). This increased immune activity observed among the male patients is of special interest since in a healthy state, men mount a lower antibody response in comparison with women [45-47]. Although the pathogenic effect of autoantibodies has not been clearly established, the presence of certain autoantibodies has been associated with organ manifestations. Noteworthy, SSA antibodies are related to pulmonary diseases [205] [206], an extraglandular manifestation we observed overrepresented in the male patients from our cohort. Further, a recent study proposed that anti-La/SSB antibodies are a risk factor associated with increased mortality in pSS patients[207]. Thus, even though the pathogenic role of pSS-associated autoantibodies remains unknown, seropositivity has a strong correlation with organ involvement and worse prognosis, supporting the conclusion that the disease course is more severe in male patients than in female patients.

Taking advantage of the large sample size, we further analyzed whether seropositivity could be related to the age at diagnosis of the patients, an indirect approach for understanding the influence of sex hormones. Indeed, sex hormones have been studied in the context of pSS[208]; sex hormones have been suggested to influence the immune system, especially in terms of antibody production[209]. To evaluate whether the number or percentage of autoantibody positive individuals diagnosed was related to menopause (the most common period for pSS diagnosis), we further stratified the female and male patients with and without autoantibodies based on age at diagnosis (**Paper IV, Figure 1**). We observed an increasing number of autoantibody-positive women being diagnosed up to 60 years of age, and that at the same time, a steadily increasing number of autoantibody negative women receiving the diagnosis (**Paper IV**, **Figure 1A**). The male group displayed a comparable pattern (**Paper IV, Figure 1B**). Consistently, also when analyzed as percent autoantibody positive (**Paper IV, Figure 1C**), the trend was similar in both the female and male group. Already in the late thirties/early forties the percentage of autoantibody positive patients diagnosed with pSS started to decline and did so steadily until the mid-seventies. Very few males were diagnosed after the age of 75 (n=2), making the last point of the curve less

relevant to consider. Altogether, the data show a consistent higher percentage of autoantibody-positive men. Neither the female nor male group did show any obvious change specific for age 50 or 55, which is commonly used as a proxy for menopause.

In relation to EGM and comorbidities, interstitial lung disease, lymphadenopathy and, notably, lymphoma were significantly more frequent in male patients (**Paper IV, Table 3**). When we characterized the malignancies diagnosed in the patients, we did not see significant differences between the sexes in regards to lymphoma classifications (**Paper IV, Table 4**). On the other hand, hypothyroidism was more common in female patients (**Paper IV, Table 3**). It is well known that pSS patients have an increased risk for developing non-Hodgkin lymphoma[210-214]. Sex-specific risk for lymphoma development in patients with rheumatic disease has been seldom studied, mainly due to the inclusion of mostly female patients. Nevertheless, Ansell *et al* have reported a significantly higher incidence of lymphoma in male RA patients. Despite the increased association of autoimmune diseases and lymphoma in men[215], earlier studies of sex differences in lymphoproliferative malignancies in pSS have not shown a clear sex-specific predominance[216 217]. In contrast, our present study is the largest pSS cohort to report a significantly increased risk for male patients to present lymphoma in comparison with female patients. This is in accordance with the results from a smaller patient sample from which an increased risk for men affected with pSS, SLE, RA and autoimmune hemolytic anemia to develop lymphoma was reported[218].

The studied cohort offers a valuable large group of clinically carefully characterized patients with pSS, allowing for analysis of parameters that differ between men and women affected by the syndrome. The long follow-up time is essential for identifying clinical manifestations at different time points of the disease course. However, the patients included in this cohort were mostly included at tertiary referral centers of university hospital clinics. A possible limitation of the study is that the study population might therefore not mirror the general pSS patient population and that the patients described in this study represent cases with an overall more severe disease phenotype, both female and male patients. A further possibility is that male patients with mild symptoms and less severe disease are less often referred, as the primary health care doctor may not be as likely to suspect Sjögren's syndrome due to its rarity in men, resulting in only men with more severe disease being included in the study. As the evaluation of extraglandular manifestations was dependent on doctors' clinical assessments and did not include specific laboratory or physiologic tests unless the patient had symptoms, it is also possible that subclinical extraglandular manifestations may have been missed. However, the mean number of extraglandular manifestations diagnosed did not differ significantly between centers, nor did the proportion

of men and women contributed. A further limitation is the lack of EULAR primary Sjögren's syndrome disease activity (ESSDAI) and patient-reported (ESSPRI) indexes[219] at diagnosis, as well as information on other common extraglandular manifestations such as neurological diseases.

A common drawback when studying a patient population in a retrospective manner is that the observations may be biased due to, for example, disease duration. In other words, more severe EGM or comorbidities could be associated with a longer time of dealing with the disease, introducing a bias in the analysis that could potentially deviate the risk for a particular manifestation with time and not another variable of interest (e.g. sex). In our present cohort, we circumvented this issue by comparing the number of years from diagnosis to last year of follow-up. The comparable years in disease duration (**Paper IV, Table 1**) between female and male pSS patients allowed us to compare the two groups without recurring to any sort of correction such as Cox regression analysis. We can, therefore, assert our observations because time has been accounted for equally in both populations.

3.5 Sex-specific clinical features in SLE and lupus nephritis

Among sex-biased rheumatic diseases, SLE is the one where more work has been done in relation to sexual dimorphism in the clinical presentation of the disease. Men with SLE have been described to exhibit more serious systemic complications, namely serositis and nephritis. Lupus nephritis is a critical pathological process that may lead to increased mortality in these patients. In **Paper V,** we analyzed sex differences in diagnosis criteria fulfillment and further studied whether there were important sex differences in the pathological features of lupus nephritis.

The group studied consisted of 1233 SLE patients (1065 women and 168 men) included in the Scandinavian DISSECT consortium cohort. The lupus nephritis investigation was conducted in a sub-cohort of the same patients, consisting of 907 patients (784 women and 123 men). After considering the frequencies of each diagnosis criterion, we observed that male patients were significantly more affected by serositis (**Paper V, Table 2**). The increased frequency of serositis in male SLE has been recognized in previous studies, where male sex has been identified as a risk factor for the development of pleuritis, but not pericarditis[150 220 221 222]. However, in our study, we found both pleuritis and pericarditis to be significantly more present in men.

Although the male susceptibility for serositis is currently not well understood, genetic polymorphisms could partly account for it. One example of how this may occur is the SNP in *CXCR3* rs34334103 described by Im *et al*[223], which is associated with pleuritis only in

male SLE patients. The CXCR3 gene, situated on the X chromosome, encodes a chemokine receptor which interacts with CXCL9, CXCL10 and CXCL11. This SNP may disrupt the chemokine axis, promoting a potential increase in lymphocyte migration into target tissues. This process might be enhanced in male SLE patients carrying this SNP and, thus, promote inflammation of the pleurae. In general, men with rheumatic diseases present more frequently with pulmonary complications. For example, patients with rheumatoid arthritis can also present with extra-articular manifestations, such as pleuritis. Rheumatoid pleuritis is more common in male than female patients[224]. As shown in **Papers III** and **IV**, men with primary Sjögren's syndrome also exhibit more frequently interstitial lung disease[166 225]. Thus, it appears that the lung is a specially affected organ in male patients with systemic autoimmunity. Further studies shall aim to clarify the possible pathophysiological mechanisms involved in this sexually dimorphic feature.

Renal involvement was prominently more common in men with SLE, as reflected by higher frequencies of proteinuria, cellular casts and, most importantly, lupus nephritis (**Paper V, Tables 2 & 3**). Currently, there are no proposed molecular mechanisms to explain this male propensity to present with renal manifestations. It is of note, though, that men from our cohort had more immunological disturbances (**Paper V, Table 2**), a criterion including positivity for autoantibodies such as anti-dsDNA antibodies, which are known to be a strong risk factor for lupus nephritis, although the difference between men and women with the regard to this parameter did not reach statistical significance. This enhanced humoral response in the male group could exacerbate the inflammation occurring in the glomeruli, contributing to the shorter time for nephritis development and progression to end-stage renal disease observed in our cohort. Also, our study is the first to identify a clear tendency of men with SLE to present APS associated nephropathy, a more severe form when compared with other types of nephritis classifications (**Paper V, Table 3**).

Overall, our studied cohort confirmed that men with SLE are more likely to develop lupus nephritis (**Paper V, Figure 1**), as well as expanded our knowledge on this complication by showing that men can be diagnosed with a more severe form of nephritis and are more prone to progress into end stage renal disease (**Paper V, Figure 1**). Since increased renal disease in male SLE is a quite established notion, one can't automatically rule out a possible bias in disease exploration. Renal function assessment in serum and urine are easy methods and, most of all, virtually non-invasive. Consistent and continued renal monitoring, thus, should help in screening renal involvement equally in both sex groups. The fact that in our cohort we didn't see sex differences in frequencies of renal biopsies

performed suggests that the procedure was made based on pathological suggestive findings rather than sex bias in clinical practice.

Conversely, female patients presented more frequently with malar rash, photosensitivity, oral ulcers and arthritis (**Paper V, Table 2**). The higher susceptibility for skin manifestations could be partly due to the effect of hormones. Although infrequent, some women may develop dermatitis during their menses, a sensitization triggered by the increased levels of estrogen during the menstrual period[226]. Estrogen may play a crucial role in skin inflammation and flares in SLE and, therefore, have a more negative impact in women due to its higher concentration than in men.

4 CONCLUSION AND FUTURE PERSPECTIVES

Sexual dimorphism occurs in the susceptibility, prevalence, course and severity of many common diseases. Systemic autoimmune diseases, specifically pSS and SLE, stand out as conditions among those with the highest gender-related differences in prevalence. Unequivocally, every report distinguishes that female are more prone to the development of these diseases. Even though this fact is apparent, we still lack an answer as to why females are more affected; in fact, the area is underexplored, especially considering its impact.

The results from our studies hereby exposed allow us to propose a model for sexual dimorphism in systemic autoimmune diseases (**Figure 6**).

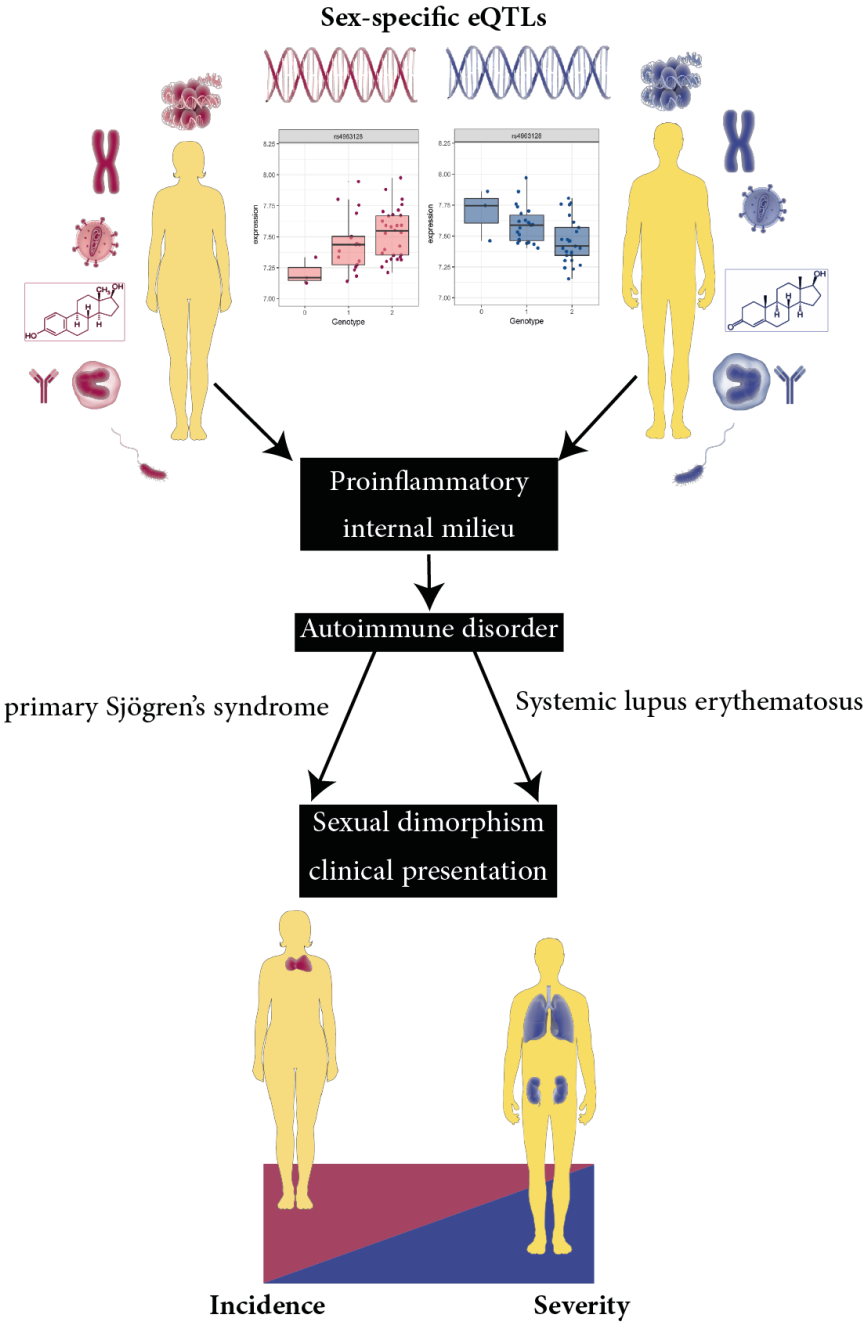


Figure 6: Sexual dimorphism in disease regulation and presentation of pSS and SLE.

The development of an AID is influenced by many factors. Although the exact magnitude of their contribution for disease pathogenesis remains elusive, some of these factors can act differently based on the sex of the individual e.g. differential gene regulation by sex-specific and cell-specific eQTLs. The combination of these intrinsic and extrinsic factors ultimately culminates in the triggering of an autoimmune process. Underlying sex differences in disease susceptibility also translate in to sex differences in disease phenotype, mainly in relation with severe systemic manifestations. In the case of pSS, sexual dimorphism can be observed more evidently at an early stage of the disease while these differences decrease at a later stage.

It is noteworthy that our results provide a comparable disease phenotype between diseases. Men with pSS and SLE present a more severe phenotype, which might not be all too surprising due to the similarity and often overlap of these two diseases. On the other hand, this common background can be problematic when trying to understand the diversity in tissue and organ manifestations. Though pSS and SLE might share a strong genetic backdrop, many have suggested differences in their etiology (e.g. different viral infections) and some clinical features are fundamentally hallmarks of each disease. For example, while pSS is characterized by exocrine gland dysfunction and an increased risk for B cell malignancies, patients with pSS do not usually present with photosensitivity and serositis which are a stamp of SLE. Rather, the common genetic susceptibility may be responsible for promoting a generalized inflammatory environment that then influences different organs in varying degrees of severity. This reinforces the idea that the dysregulation observed in AID might not only be exclusive of the immune system, but also in the tissue-specific milieu where these cells are acting on. Further, this predilection for certain organs may be because susceptible tissues may themselves been under the influence of other factors that display a sexual dimorphic pattern, resulting in a sex-specific organ involvement.

Although men with rheumatic diseases have been for long considered as having a more severe disease, our results raise questions about how we are approaching and handling this typical understudied group of patients. There are many reasons that we could take into account in order to explain this feature: sex bias in clinical evaluation, sex bias in healthcare attitudes, underdiagnosis of men with rheumatic diseases, to name a few. In spite of this, the biological evidence presented in this thesis sustains that organ involvement and deterioration, hematological malignancies and autoantibody positivity are more frequent in men, phenotypical traits which might not necessarily be associated with a delay in diagnosis but instead to an inherent immunologically suppressed state that, upon major dysregulation,

is not able to counteract the overwhelming effects of the autoimmune process and manifests more systemically than compared to women.

This thesis aimed to improve our understanding of the molecular mechanisms that govern this sexual dimorphism. We could take advantage of this knowledge to envision sex-specific therapies that might target sex-specific dysregulated genes or molecular pathways. Our findings would also be important for other biological fields and for clinical studies, where sex bias is often disregarded. Finally, a sex-specific approach to disease is a step further towards what is called the future of patient care: Precision Medicine.

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